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5.1195

U S APPLICATION NO (If known, see 37 CFR 1.5)

10/018349

**TRANSMITTAL LETTER TO THE UNITED STATES  
DESIGNATED/ELECTED OFFICE (DO/EO/US)  
CONCERNING A FILING UNDER 35 U.S.C. 371**

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/JP00/04140	23 June 2000	24 June 1999

## TITLE OF INVENTION

METHOD OF INHIBITING LEAKAGE OF DRUG ENCAPSULATED IN LIPOSOMES

## APPLICANT(S) FOR DO/EO/US

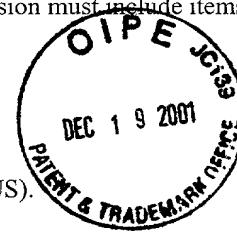
Yasuki Kato, et al.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1.  This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2.  This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3.  This express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.
4.  The US has been elected by the expiration of 19 months from the priority date (Article 31).
5.  A copy of the International Application as filed (35 U.S.C. 371(c)(2))
  - a.  is transmitted herewith (required only if not transmitted by the International Bureau).
  - b.  has been transmitted by the International Bureau.
  - c.  is not required, as the application was filed in the United States Receiving Office (RO/US).
6.  A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7.  Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
  - a.  are transmitted herewith (required only if not transmitted by the International Bureau).
  - b.  have been transmitted by the International Bureau.
  - c.  have not been made; however, the time limit for making such amendments has NOT expired.
  - d.  have not been made and will not be made.
8.  A translation of the amendments to the claims under PCT Article 19 into English (35 U.S.C. 371(c)(3)).
9.  An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10.  A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 into English (35 U.S.C. 371(c)(5)).

## Items 11 to 20 below concern other document(s) or information included:

11.  An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12.  An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13.  A FIRST preliminary amendment.
14.  A SECOND or SUBSEQUENT preliminary amendment.
15.  A substitute specification.
16.  A change of power of attorney and/or address letter.
17.  A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
18.  A second copy of the published international application under 35 U.S.C. 154(d)(4).
19.  A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
20.  Other items or information: Copies of: Form PCT/RO/101, Form PCT/ISA/210, Form PCT/IB/301, Form PCT/IB/304 and Form PCT/IB308.



21.  The following fees are submitted:**Basic National Fee (37 CFR 1.492(a)(1)-(5)):**

Search Report has been prepared by the EP or JPO ..... \$890.00  
 International preliminary examination fee paid to USPTO  
 (37 CFR 1.492(a)(1)) ..... \$710.00  
 No international preliminary examination fee paid to USPTO (37 CFR 1.492  
 (a)(1)) but international search fee paid to USPTO (37 CFR 1.492(a)(2)) ..... \$740.00  
 Neither international preliminary examination fee (37 CFR 1.492(a)(1))  
 nor international search fee (37 CFR 1.492(a)(2)) paid to USPTO ..... \$1,040.00  
 International preliminary examination fee paid to USPTO (37 CFR 1.492  
 (a)(4)) and all claims satisfied provisions of PCT Article 33(1)-(4) ..... \$100.00

**ENTER APPROPRIATE BASIC FEE AMOUNT =**

\$890.00

Surcharge of **\$130.00** for furnishing the oath or declaration later than  20  30 months  
 from the earliest claimed priority date (37 CFR 1.492(e)).

\$

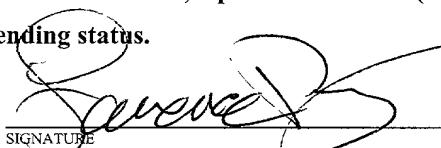
Claims	Number Filed	Number Extra	Rate	
Total Claims	53-20 =	33	X \$18.00	\$594.00
Independent Claims	8 - 3 =	5	X \$84.00	\$420.00
Multiple dependent claim(s) (if applicable)			+ \$280.00	\$280.00
<b>TOTAL OF ABOVE CALCULATIONS =</b>				\$2184.00
<input type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by ½.		+	\$	
<b>SUBTOTAL =</b>				\$2184.00
Processing fee of <b>\$130.00</b> for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)).			\$	
<b>TOTAL NATIONAL FEE =</b>				\$
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). <b>\$40.00</b> per property +			\$40.00	
<b>TOTAL FEES ENCLOSED =</b>				\$2224.00
				<b>Amount to be:</b>
				<b>refunded</b> \$
				<b>charged</b> \$

a.  A check in the amount of \$ \_\_\_\_\_ over the above fees is enclosed.  
 b.  Please charge my Deposit Account No. \_\_\_\_\_ in the amount of \$ \_\_\_\_\_ to cover the above fees. A duplicate copy of this sheet is enclosed.  
 c.  The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 06-1205. A duplicate copy of this sheet is enclosed.  
 d.  Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

**NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.**

SEND ALL CORRESPONDENCE TO:

Lawrence S. Perry  
 FITZPATRICK, CELLA, HARPER & SCINTO  
 30 Rockefeller Plaza  
 New York, NY 10112  
 Tel: (212) 218-2100  
 Fax: (212) 218-2200

  
 SIGNATURE  
 Lawrence S. Perry  
 NAME  
 31,865  
 REGISTRATION NUMBER

5.1195

PATENT APPLICATION

## IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: )  
YASUKI KATO, ET AL. )      Examiner: Not Yet Assigned  
Application No.: Not Yet Assigned )      Group Art Unit: Not Yet Assigned  
National Phase of PCT Application No. )  
PCT/JP00/04140 filed June 23, 2000 )  
Filed: Currently herewith )  
For:      METHOD OF INHIBITING :  
              LEAKAGE OF DRUG :  
              ENCAPSULATED IN LIPOSOMES      December 18, 2001

Commissioner for Patents  
Washington, D.C. 20231

PRELIMINARY AMENDMENT

Sir:

Prior to action on the merits, please amend the above-identified application as follows:

IN THE SPECIFICATION

Please substitute the paragraph starting at page 1, line 17 and ending at page 2, line 24 with the following replacement paragraph. A marked-up copy of this paragraph, showing the changes made thereto, is attached.

Bally et al. has found a method of inhibiting the leakage of an antitumor agent from liposomes (Japanese Patent No. 2,572,554). According to the method, a transmembrane

potential is generated by providing a concentration gradient of a charged substance inside and outside of liposomes and a drug which can be ionized is encapsulated in the liposomes due to a pH gradient or a Na<sup>+</sup>/K<sup>+</sup> concentration gradient to thereby inhibit the leakage of a drug from the liposomes. As a method of encapsulating a drug in liposomes and inhibiting the leakage thereof similarly using a pH gradient, Barenholz et al. have invented a method using a pH gradient inside and outside of liposomes which is achieved by an ammonium ion gradient using ammonium sulfate (Japanese Patent No. 2,659,136). Both of these methods are not restricted in the particle size of the liposomes to be used, and these liposomes involve small unilamellar vesicles (SUVs), large unilamellar vesicles (LUVs), multilamellar vesicles (MLVs) and the like. On the other hand, Maurer et al. reported that when ciprofloxacin was encapsulated in LUVs of 190 nm in an average particle size by the method under a pH gradient using ammonium sulfate, ciprofloxacin rapidly leaked out of the LUVs in 50% mouse serum at 37°C (*Biochim. Biophys. Acta*, 1374, 9 (1998)). According to this report, ciprofloxacin was not crystallized (precipitated) in the liposomes, different from doxorubicin or the like, and thus leaked out. Thus, the methods presented by the two patents as described above are not necessarily the most desirable methods from the viewpoint of the leakage of drugs encapsulated in liposomes. Therefore, further improvement has been required.

Please substitute the paragraph at page 9, lines 12-20 with the following replacement paragraph. A marked-up copy of this paragraph, showing the changes made thereto, is attached.

Examples of the antibiotic include minocycline, tetracycline, piperacillin sodium, sultamicillin tosylate, amoxicilline, ampicillin, bacampicillin, aspoxicilin, cefdinir, flomoxef sodium, cefotiam, cefcapene pivoxil, cefaclor, cefteram pivoxil, cephazolin sodium, cefradine, clarithromycin, clindamycin, erythromycin, levofloxacin, tosufloxacin tosylate, ofloxacin, ciprofloxacin, arbekacin, isepamicin, dibekacin, amikacin, gentamicin, vancomycin, fosfomycin, derivatives thereof, and the like.

IN THE CLAIMS:

Please amend Claims 1-16 and 19-25 to read as follows. A marked-up copy of these claims, showing the changes made thereto, is attached.

1. (Amended) A method of preparing a drug encapsulated in liposomes, which comprises selecting a drug and encapsulating said drug using at least two lipid bilayers of the liposomes.

2. (Amended) A method of preparing a drug encapsulated in liposomes, which comprises selecting a drug and encapsulating said drug using lipid having a phase transition temperature higher than *in vivo* temperature as lipid constituting the liposomes.

3. (Amended) A method of preparing a drug encapsulated in liposomes, which comprises selecting a drug and encapsulating said drug satisfying at least two requirements selected from the group consisting of: using at least two lipid bilayers of the liposomes, controlling the average particle size of the liposomes to 120 nm or more, and using lipid having a phase transition temperature higher than *in vivo* temperature as lipid constituting the liposomes.

4. (Amended) The method according to claim 2 or 3, wherein the lipid comprises at least one component selected from the group consisting of hydrogenated soybean phosphatidylcholine, polyethylene glycol-modified phospholipid, and cholesterol.

5. (Amended) The method according to claim 2 or 3, wherein the lipid comprises at least one component selected from the group consisting of distearoyl phosphatidylcholine, polyethylene glycol-modified phospholipid, and cholesterol.

6. (Amended) The method according to claim 3, which comprises using at least two lipid bilayers of the liposomes, and controlling the average particle size of the liposomes to 120 nm or more.

7. (Amended) The method according to claim 3 or 6, wherein the liposomes have an average particle size of 120 to 500 nm.

8. (Amended) The method according to any one of claims 1 to 3 or 6, wherein

the biological component is a blood component.

9. (Amended) The method according to claim 8, wherein the drug encapsulated is an indolocarbazole derivative.

10. (Amended) The method according to claim 8, wherein the drug encapsulated is an antitumor agent.

11. (Amended) The method according to claim 8, wherein the drug encapsulated is an antibiotic.

12. (Amended) The method according to claim 8, wherein the drug encapsulated is a pharmaceutically active substance.

13. (Amended) A liposome preparation comprising encapsulated drug, at least two lipid bilayers, and having an average particle size of 120 nm or more.

14. (Amended) A liposome preparation comprising encapsulated drug, at least two lipid bilayers wherein the lipid constituting the liposomes has a phase transition temperature higher than *in vivo* temperature.

15. (Amended) A liposome preparation comprising an encapsulated drug, and

wherein the liposomes have an average particle size of 120 nm or more, and the lipid constituting the liposomes has a phase transition temperature higher than *in vivo* temperature.

16. (Amended) A liposome preparation comprising an encapsulated drug, wherein said liposome satisfies at least two requirements selected from the group consisting of: the number of lipid bilayers of the liposomes is at least two, the liposomes have an average particle size of 120 nm or more, and lipid constituting the liposomes has a phase transition temperature higher than *in vivo* temperature.

19. (Amended) The liposome preparation according to any one of claims 14 to 16, wherein the lipid comprises at least one component selected from the group consisting of hydrogenated soybean phosphatidylcholine, polyethylene glycol-modified phospholipid, and cholesterol.

20. (Amended) The liposome preparation according to any one of claims 14 to 16, wherein the lipid comprises at least one component selected from the group consisting of distearoyl phosphatidylcholine, polyethylene glycol-modified phospholipid, and cholesterol.

21. (Amended) The liposome preparation according to any one of claims 13, 15 or 16, wherein the liposomes have an average particle size of 120 to 500 nm.

22. (Amended) The liposome preparation according to claim 21, wherein the

drug encapsulated is an indolocarbazole derivative.

23. (Amended) The liposome preparation according to claim 21, wherein the drug encapsulated is an antitumor agent.

24. (Amended) The liposome preparation according to claim 21, wherein the drug encapsulated is an antibiotic.

25. (Amended) The liposome preparation according to claim 21, wherein the drug encapsulated is a pharmaceutically active substance.

REMARKS

The claims have been amended to correct their dependency and conformity with accepted U.S. practice and the specification has been changed to correct typographical errors. No new matter has been added.

Entry hereof is earnestly solicited.

Applicants' undersigned attorney may be reached in our New York office by telephone at (212) 218-2100. All correspondence should continue to be directed to our below listed address.

Respectfully submitted,



Lawrence S. Perry  
Attorney for Applicants  
Registration No. 31,865

FITZPATRICK, CELLA, HARPER & SCINTO  
30 Rockefeller Plaza  
New York, New York 10112-3801  
Facsimile: (212) 218-2200

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NY\_MAIN 224135 v1

(National Phase of PCT Application No.PCT/JP00/04140)  
Attorney Docket No. 5.1195

VERSION WITH MARKINGS TO SHOW CHANGES MADE TO CLAIMS

1. (Amended) A method of [inhibiting the leakage of] preparing a drug encapsulated in liposomes [in the presence of a biological component], which comprises selecting a drug and encapsulating said drug using at least two lipid bilayers of the liposomes.

2. (Amended) A method of [inhibiting the leakage of] preparing a drug encapsulated in liposomes [in the presence of a biological component], which comprises selecting a drug and encapsulating said drug using lipid having a phase transition temperature higher than *in vivo* temperature as lipid constituting the liposomes.

3. (Amended) A method of [inhibiting the leakage of] preparing a drug encapsulated in liposomes [in the presence of a biological component], which comprises selecting a drug and encapsulating said drug satisfying at least two requirements selected from the group consisting of [the following three requirements]: using at least two lipid bilayers of the liposomes, controlling the average particle size of the liposomes to 120 nm or more, and using lipid having a phase transition temperature higher than *in vivo* temperature as lipid constituting the liposomes.

4. (Amended) The method [of inhibiting the leakage] according to claim 2 or 3, wherein the lipid comprises at least one component selected from the group consisting of hydrogenated soybean phosphatidylcholine, polyethylene glycol-modified phospholipid, and cholesterol.

5. (Amended) The method [of inhibiting the leakage] according to claim 2 or 3, wherein the lipid comprises at least one component selected from the group consisting of distearoyl phosphatidylcholine, polyethylene glycol-modified phospholipid, and cholesterol.

6. (Amended) [A] The method [of inhibiting the leakage of a drug encapsulated in liposomes in the presence of a biological component] according to claim 3, which comprises using at least two lipid bilayers of the liposomes, and controlling the average particle size of the liposomes to 120 nm or more.

7. (Amended) The method [of inhibiting the leakage] according to claim 3 or 6, wherein the liposomes have an average particle size of 120 to 500 nm.

8. (Amended) The method [of inhibiting the leakage] according to any one of claims 1 to [7] 3 or 6, wherein the biological component is a blood component.

9. (Amended) The method [of inhibiting the leakage] according to [any one

of] claim[s 1 to] 8, wherein the drug encapsulated is an indolocarbazole derivative.

10. (Amended) The method [of inhibiting the leakage] according to [any one of] claim[s 1 to] 8, wherein the drug encapsulated is an antitumor agent.

11. (Amended) The method [of inhibiting the leakage] according to [any one of] claim[s 1 to] 8, wherein the drug encapsulated is an antibiotic.

12. (Amended) The method [of inhibiting the leakage] according to [any one of] claim[s 1 to] 8, wherein the drug encapsulated is a pharmaceutically active substance.

13. (Amended) A liposome preparation comprising encapsulated drug, at least two [in which the number of] lipid bilayers [of the liposomes is at least two], and [the liposomes have] having an average particle size of 120 nm or more.

14. (Amended) A liposome preparation comprising encapsulated drug, at least two [in which the number of] lipid bilayers [of the liposomes is at least two, and] wherein the lipid constituting the liposomes has a phase transition temperature higher than *in vivo* temperature.

15. (Amended) A liposome preparation [in which the] comprising an

encapsulated drug, and wherein the liposomes have an average particle size of 120 nm or more, and the lipid constituting the liposomes has a phase transition temperature higher than *in vivo* temperature.

16. (Amended) A liposome preparation comprising an encapsulated drug, wherein said liposome [which] satisfies at least two requirements selected from the group consisting of [the following three requirements]: the number of lipid bilayers of the liposomes is at least two, the liposomes have an average particle size of 120 nm or more, and lipid constituting the liposomes has a phase transition temperature higher than *in vivo* temperature.

19. (Amended) The liposome preparation according to any one of claims 14 to [18] 16, wherein the lipid comprises at least one component selected from the group consisting of hydrogenated soybean phosphatidylcholine, polyethylene glycol-modified phospholipid, and cholesterol.

20. (Amended) The liposome preparation according to any one of claims 14 to [18] 16, wherein the lipid comprises at least one component selected from the group consisting of distearoyl phosphatidylcholine, polyethylene glycol-modified phospholipid, and cholesterol.

21. (Amended) The liposome preparation according to any one of claims 13 [and] 15 [to 18] or 16, wherein the liposomes have an average particle size of 120 to 500 nm.

22. (Amended) The liposome preparation according to [any one of] claim[s 13 to] 21, wherein the drug encapsulated is an indolocarbazole derivative.

23. (Amended) The liposome preparation according to [any one of] claim[s 13 to] 21, wherein the drug encapsulated is an antitumor agent.

24. (Amended) The liposome preparation according to [any one of] claim[s 13 to] 21, wherein the drug encapsulated is an antibiotic.

25. (Amended) The liposome preparation according to [any one of] claim[s 13 to] 21, wherein the drug encapsulated is a pharmaceutically active substance.

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SPECIFICATION

METHOD OF INHIBITING LEAKAGE OF  
DRUG ENCAPSULATED IN LIPOSOMES

TECHNICAL FIELD

The present invention relates to a method of inhibiting the leakage of a drug encapsulated in liposomes and liposome preparations which are stable *in vivo*.

BACKGROUND ART

It has already been a practice in the medical field to encapsulate drugs in liposomes and thus enhance the drug effects. The technique has been clinically applied mainly by the injection method. In intravascular administration among injection operations, it is important for enhancing the therapeutic effect that a drug encapsulated in liposomes remains in the liposomes over a relatively long period of time without leakage.

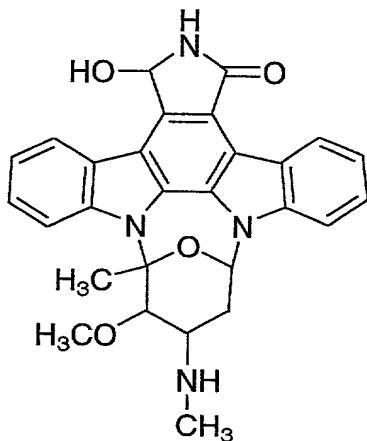
B. has found a method of inhibiting the leakage of an antitumor agent from liposomes (Japanese Patent No. 2,572,554). According to the method, a transmembrane potential is generated by providing a concentration gradient of a charged substance inside and outside of liposomes and a drug which can be ionized is encapsulated in the liposomes due to a pH gradient or a  $\text{Na}^+/\text{K}^+$

concentration gradient to thereby inhibit the leakage of a drug from the liposomes. As a method of encapsulating a drug in liposomes and inhibiting the leakage thereof similarly using a pH gradient, Barenholz et al. have invented a method using a pH gradient inside and outside of liposomes which is achieved by an ammonium ion gradient using ammonium sulfate (Japanese Patent No. 2,659,136). Both of these methods are not restricted in the particle size of the liposomes to be used, and these liposomes involve small unilamellar vesicles (SUVs), large unilamellar vesicles (LUVs), multilamellar vesicles (MLVs) and the like. On the other hand, Maurer et al. reported that when ciprofloxacin was encapsulated in LUVs of 190 nm in an average particle size by the method under a pH gradient using ammonium sulfate, ciprofloxacin rapidly leaked out of the LUVs in 50% mouse serum at 37°C (*Biochim. Biophys. Acta*, 1374, 9 (1998)). According to this report, ciprofloxacin was not crystallized (precipitated) in the liposomes, different from doxorubicin or the like, and thus leaked out. Thus, the methods presented by the two patents as described above are not necessarily the most desirable methods from the viewpoint of the leakage of drugs encapsulated in liposomes. Therefore, further improvement has been required.

DISCLOSURE OF THE INVENTION

An object of the present invention is to provide a method of inhibiting the leakage of a drug encapsulated in liposomes, and liposome preparations which are stable *in vivo*.

The inventors previously found that liposome preparations in which an indolocarbazole derivative, such as UCN-01 or the like, is encapsulated have improved stability and the like *in vivo* (WO97/48398).



UCN-01

As the results of subsequent studies, the inventors have found that the leakage of a drug can be efficiently inhibited by controlling the average particle size of liposomes to 120 nm or more or using at least two lipid bilayers of the liposomes. Furthermore, they have found that the leakage of a drug can be inhibited by using a component having a phase transition temperature higher than

in vivo temperature as a component constituting the lipid bilayers.

Specifically, the present invention relates to a method of inhibiting the leakage of a drug encapsulated in liposomes in the presence of a biological component, which comprises using at least two lipid bilayers of the liposomes, or a method of inhibiting the leakage of a drug encapsulated in liposomes in the presence of a biological component, which comprises using lipid having a phase transition temperature higher than *in vivo* temperature as lipid constituting the liposomes.

Furthermore, the present invention relates to a method of inhibiting the leakage of a drug encapsulated in liposomes in the presence of a biological component, which comprises satisfying at least two requirements selected from the group consisting of the following three requirements: using at least two lipid bilayers of the liposomes, controlling the average particle size of the liposomes to 120 nm or more, and using lipid having a phase transition temperature higher than *in vivo* temperature as lipid constituting the liposomes.

Moreover, the present invention relates to a method of inhibiting the leakage of a drug encapsulated in liposomes in the presence of a biological component, which comprises using at least two lipid bilayers of the

liposomes, and controlling the average particle size of the liposomes to 120 nm or more.

Also, the present invention provides a liposome preparation in which the number of lipid bilayers of the liposomes is at least two, and the liposomes have an average particle size of 120 nm or more, a liposome preparation in which the number of lipid bilayers of the liposomes is at least two, and lipid constituting the liposomes has a phase transition temperature higher than *in vivo* temperature, or a liposome preparation in which the liposomes have an average particle size of 120 nm or more, and lipid constituting the liposomes has a phase transition temperature higher than *in vivo* temperature.

Furthermore, the present invention provides a liposome preparation which satisfies at least two requirements selected from the group consisting of the following three requirements: the number of lipid bilayers of the liposomes is at least two, the liposomes have an average particle size of 120 nm or more, and lipid constituting the liposomes has a phase transition temperature higher than *in vivo* temperature.

Each of the liposome preparations as described above can inhibit the leakage of a drug encapsulated in liposomes in the presence of a biological component.

Examples of the lipid constituting the liposomes include phospholipid, glyceroglycolipid, sphingoglycolipid,

cholesterol, and the like. Particularly, phospholipid is preferably used. Among these, it is preferable to use lipid having a phase transition temperature higher than *in vivo* temperature (35 to 37°C). The lipid may be modified by a nonionic surfactant such as polysorbate 80, Pluronic F68, etc.; a cationic surfactant such as benzalkonium chloride etc.; an anionic surfactant such as sodium laurylsulfate etc.; a polysaccharide such as dextran etc., or a derivative thereof; a polyoxyethylene derivative such as polyoxyethylene lauryl alcohol, polyethylene glycol, etc.; or the like.

Examples of the phospholipid include natural or synthetic phospholipids, such as phosphatidylcholine (soybean phosphatidylcholine, yolk phosphatidylcholine, distearoyl phosphatidylcholine, dipalmitoyl phosphatidylcholine, etc.), phosphatidylethanolamine (distearoyl phosphatidylethanolamine, dipalmitoyl phosphatidylethanolamine, etc.), phosphatidylserine, phosphatidic acid, phosphatidylglycerol, phosphatidylinositol, lysophosphatidylcholine, sphingomyelin, polyethylene glycol-modified phospholipid, yolk lecithin, soybean lecithin, hydrogenated phospholipid, etc.; and the like. Among these, it is preferable to use phospholipid having a phase transition temperature higher than *in vivo* temperature (35 to 37°C) (for example,

distearoyl phosphatidylcholine, dipalmitoyl  
phosphatidylethanolamine, *N*-stearoyl sphingomyelin, etc.)

Examples of the glyceroglycolipid include sulfoxyribosylglyceride, diglycosyldiglyceride, digalactosyldiglyceride, galactosyldiglyceride, glycosyldiglyceride, and the like. Among these, it is preferable to use glyceroglycolipid having a phase transition temperature higher than *in vivo* temperature (35 to 37°C) (for example, 1,2-*O*-dipalmitoyl-3-*O*- $\beta$ -D-glucuronosyl-sn-glycerol, 1,2-*O*-distearoyl-3-*O*- $\beta$ -D-glucuronosyl-sn-glycerol, etc.)

Examples of the sphingoglycolipid include galactosylcerebroside, lactosylcerebroside, ganglioside, and the like. Among these, it is preferable to use sphingoglycolipid having a phase transition temperature higher than *in vivo* temperature (35 to 37°C) (for example, *N*-stearoyldihydrogalactosylsphingosine, *N*-stearoyldihydrolactosylsphingosine, etc.)

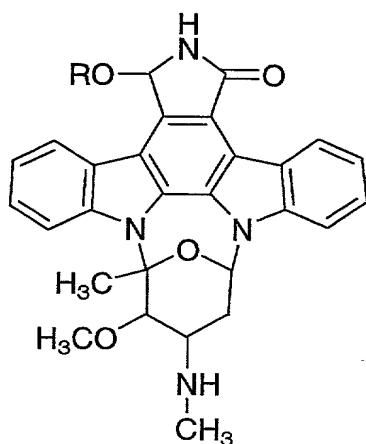
These lipids may be used alone or in combination. When the lipids are used in combination, lipid comprising at least two components selected from hydrogenated soybean phosphatidylcholine, polyethylene glycol-modified phospholipid and cholesterol, lipid comprising at least two components selected from distearoyl phosphatidylcholine, polyethylene glycol-modified phospholipid and cholesterol, or the like is used as the lipid. As the phospholipid in

the polyethylene glycol-modified phospholipid as described herein, phosphatidylethanolamine, such as distearoyl phosphatidylethanolamine or the like, is preferably used.

If necessary, it is possible to use, together with the lipid component, a membrane-stabilizing agent, for example, a sterol such as cholesterol etc.; an antioxidant such as tocopherol etc.; a charged substance such as stearylamine, dicetyl phosphate, ganglioside, etc.

Examples of the drug to be encapsulated in liposomes include indolocarbazole derivatives, an antitumor agent, an antibiotic, an antifungal agent, a pharmaceutically active substance, and the like.

Examples of the indolocarbazole derivatives include UCN-01, derivatives thereof (for example, the following compounds), and the like:



wherein R represents hydrogen or lower alkyl.

The lower alkyl in the definition of R means linear or branched alkyl having 1 to 6 carbon atoms such as methyl, ethyl, propyl, isopropyl, sec-butyl, tert-butyl, pentyl, hexyl, or the like.

Examples of the antitumor agent include actinomycin D, mitomycin C, chromomycin, doxorubicin, epirubicin, vinorelbine, daunorubicin, aclarubicin, bleomycin, peplomycin, vincristine, vinblastine, vindesine, etoposide, methotrexate, 5-Fu, tegafur, cytarabine, enocitabine, ancitabine, taxol, taxotere, cisplatin, cytosine arabinoside, irinotecan, derivatives thereof, and the like.

Examples of the antibiotic include minocycline, tetracycline, piperacillin sodium, sultamicillin tosylate, amoxicilline, ampicillin, bacampicillin, aspocicilin, cefdinir, flomoxef sodium, cefotiam, cefcapene pivoxil, cefaclor, ceftoren pivocil, cephazolin sodium, cefozoran, clarithromycin, clindamycin, erythromycin, levofloxacin, tosufloxacin tosylate, ofloxacin, ciprofloxacin, arbekacin, isepamicin, dibekacin, amikacin, gentamicin, vancomycin, fosfomycin, derivatives thereof, and the like.

Examples of the antifungal agent include fluconazole, itraconazole, terbinafine, amphotericin B, miconazole, derivatives thereof, and the like.

Examples of the pharmaceutically active substance include a hormone, an enzyme, a protein, a peptide, an

amino acid, a nucleic acid, a gene, a vitamin, a saccharide, lipid, a synthetic drug, and the like.

Examples of the biological component include a blood component and the like.

Next, a method of producing the liposome preparations according to the present invention will be described.

The liposome preparations of the present invention can be produced by using known methods for producing liposome preparations. Examples of these known methods for producing liposome preparations include a method of preparing liposomes reported by Bangham et al. (*J. Mol. Biol.*, 13, 238 (1965)), an ethanol injection method (*J. Cell. Biol.*, 66, 621 (1975)), a French press method (*FEBS Lett.*, 99, 210 (1979)), a freezing and thawing method (*Arch. Biochem. Biophys.*, 212, 186 (1981)), a reversed phase evaporation method (*Proc. Natl. Acad. Sci. USA*, 75, 4194 (1978)), a pH gradient method (Japanese Patent No. 2,572,554, Japanese Patent No. 2,659,136, etc.)), and the like.

The pH gradient method has a number of advantages such that a high drug-encapsulation ratio in liposomes can be achieved, and that little organic solvent remains in the liposome suspension. For example, the lipid is dissolved in a solvent such as ethanol or the like, the resultant mixture is placed into a round bottomed flask, and the

solvent is evaporated under reduced pressure to thereby form a thin lipid film. Then, an acidic buffer (for example, citrate buffer) is added thereto, followed by shaking, to thereby form large MLVs. Next, the average particle size of the liposomes is controlled to the desired level (for example, 130 nm) by an extrusion method or the like. After a weakly acidic solution of a drug such as UCN-01 or the like is added to the liposome suspension, a suitable pH regulator (e.g., aqueous sodium hydroxide) is added thereto to raise the pH of the liposome suspension to around the neutral pH (the difference between the pH of the liposome suspension before and after the rise of pH is preferably 3 or more). By the above operation, the drug can be quantitatively encapsulated in the liposomes.

If necessary, it is also possible to modify the surface of the liposomes using a nonionic surfactant, a cationic surfactant, an anionic surfactant, a polysaccharide or a derivative thereof, a polyoxyethylene derivative, or the like (*Stealth Liposomes*, ed. by D.D. Lasic and F. Martin, CRC Press Inc., Florida, pp. 93-102, 1995). For the application to targeting, it is also possible to modify the surface of the liposomes with an antibody, a protein, a peptide, a fatty acid, or the like (*Stealth Liposomes*, ed. by D.D. Lasic and F. Martin, CRC Press Inc., Florida, pp. 93-102, 1995).

In addition to water, examples of the solution in which the liposomes are suspended include an acid, an alkali, various buffers, physiological saline, an amino acid infusion, and the like. Furthermore, an antioxidant such as citric acid, ascorbic acid, cysteine, ethylenediaminetetraacetic acid (EDTA), or the like, or an isotonic agent such as glycerol, glucose, sodium chloride, or the like, may be added to the liposome suspension.

Alternatively, liposomes can be formed by dissolving a drug and lipid in an organic solvent such as ethanol or the like, evaporating the solvent, and then adding physiological saline or the like thereto, followed by shaking under stirring.

The average particle size of the liposomes is preferably 120 nm or more, more preferably 120 to 500 nm. The average particle size can be controlled by, for example, the extrusion method as mentioned above.

Examples of a method of providing at least two lipid bilayers of the liposomes include the extrusion method using a membrane filter having relatively large pores (0.2  $\mu$ m, 0.4  $\mu$ m or above), a method of mechanically grinding large MLVs (using a Manton-Gorlin, a microfluidizer, or the like) (ed. and written by R.H. Muller, S. Benita and B. Bohm, "Emulsion and Nanosuspensions for the Formulation of Poorly Soluble Drugs", *High-Pressure Homogenization Techniques for the Production of Liposome*

*Dispersions: Potential and Limitations*, M. Brandl, pp. 267-294, 1998 (Scientific Publishers Stuttgart, Germany)), and the like.

The liposome preparation obtained by the above method or the like can be used as such. Alternatively, it may be mixed with a filler such as mannitol, lactose, glycine, or the like, and then freeze-dried, depending on the purpose of use, storage conditions, or the like. It is also possible to add a freeze-drying agent, such as glycerine or the like, thereto, followed by freeze-drying.

Although the liposome preparations obtained by the present invention are generally used as an injection, these may also be used as an oral preparation, a nasal preparation, an eye drop, a percutaneous preparation, a suppository, an inhalant, or the like by manufacturing the preparation into such forms.

The liposome preparations obtained by the present invention are prepared in order to stabilize a drug in a biological component (for example, a blood component), to reduce side effects and to increase accumulation in tumors.

Next, the effects of the present invention will be described by reference to the following Test Example.

#### Test Example 1

In order to monitor the leakage of UCN-01 encapsulated in liposomes in human AGP-containing rat

plasma (human AGP: 0.5 mg/mL) with the lapse of time, 0.1 mL of the UCN-01-containing liposome suspensions prepared in Examples 1 to 4 and Comparative Example 1 to 3 were each mixed with 0.9 mL of distilled water. To 0.05 mL of the resultant mixture, 4.95 mL of the rat plasma containing 0.5 mg/mL human AGP was added and mixed to obtain a liquid sample. Immediately after mixing, and after storing at 37°C for 3 hours, 2 mL of the liquid sample was subjected to gel filtration (Sephadex CL-6B, 20 mm in diameter × 20 cm, mobile phase: PBS (phosphate-buffered saline), amount of sample added: 2 mL, fraction collection amount: about 4 mL). After separating the liposome fraction from the protein fraction, 0.8 mL of 2-propanol was added per 0.4 mL of the eluate, followed by shaking. Then, the resultant mixture was centrifuged (12,000 × g, 10 minutes) at 4°C, and 20 µL of the supernatant was analyzed by high performance liquid chromatography (HPLC) under the following conditions.

**HPLC analysis conditions:**

**Column:**

YMC-Pack ODS-AM AM-312 150 mm × 6 mm (YMC)

**Mobile phase:**

A 0.1% triethylamine-containing 0.05 mol/L phosphate buffer (pH 7.3) : acetonitrile = 1:1  
(parts by volume)

Flow rate:

1.0 mL/min

Column retention temperature:

25°C

Detection:

Excitation wavelength 310 nm, fluorescence  
wavelength 410 nm

The remaining ratio of UCN-01 in liposomes was calculated in accordance with the following equation by determining the UCN-01 content in the liposome fraction and then correcting it with the use of the recovery (i.e., the sum of UCN-1 in the liposome fraction and the protein fraction) in the gel filtration  $((A+B)/C)$ :

UCN-01 content (%) in liposome fraction =  $(A/C) \times 100$

UCN-01 content (%) in protein fraction =  $(B/C) \times 100$

A: the amount of UCN-01 contained in the liposome fraction.

B: the amount of UCN-01 contained in the protein fraction.

C: the amount of UCN-01 contained in the liposome suspension subjected to gel filtration.

Remaining ratio (%) of UCN-01 in liposomes

= (UCN-01 content (%) in liposome fraction/recovery  
(%) in gel filtration)  $\times 100$

The results are shown in Table 1.

Table 1: Remaining ratio (%) of UCN-01 in liposomes

		UCN-01 remaining ratio (%)
Example 1	Immediately after mixing	95
	After 3 hours	80
Example 2	Immediately after mixing	91
	After 3 hours	57
Example 3	Immediately after mixing	94
	After 3 hours	63
Example 4	Immediately after mixing	99
	After 3 hours	81
Comparative Example 1	Immediately after mixing	90
	After 3 hours	37
Comparative Example 2	Immediately after mixing	23
	After 3 hours	0
Comparative Example 3	Immediately after mixing	93
	After 3 hours	5

Next, Examples and Comparative Examples of the present invention will be given.

#### BEST MODE FOR CARRYING OUT THE INVENTION

##### Example 1

To 5 g of hydrogenated soybean phosphatidylcholine {phase transition temperature: 58°C (*FEBS Lett.*, 386, 247-251 (1996))} was added 25 mL of a 100 mmol/L citrate buffer (pH 4.0), followed by shaking under stirring with a vortex mixer. The suspension was passed through a polycarbonate membrane filter (0.4 µm) 10 times at 70°C. Then, a 100 mmol/L citrate buffer was added thereto to give a liposome

suspension having a concentration of hydrogenated soybean phosphatidylcholine of 62.5 mg/mL. Separately, 10 mg of UCN-01 was taken and 8 mL of the liposome suspension prepared above was added thereto. The pH of the resultant mixture was adjusted to 8 by adding an appropriate amount of 1 mol/L aqueous sodium hydroxide, and then distilled water was added thereto to give a total volume of 10 mL. The mixture was heated at 70°C for 5 minutes to thereby encapsulate UCN-01 in liposomes.

The average particle size of the liposomes measured by the dynamic light scattering (DLS) method (A model DLS-700, Otsuka Electronics Ltd.; the same applies hereinafter) was 186 nm.

#### Example 2

To 5 g of hydrogenated soybean phosphatidylcholine {phase transition temperature: 58°C (*FEBS Lett.*, 386, 247-251 (1996))} was added 25 mL of a 100 mmol/L citrate buffer (pH 4.0), followed by shaking under stirring with a vortex mixer. The suspension was passed through a polycarbonate membrane filter (0.4 µm) twice at 70°C, and further passed through a polycarbonate membrane filter (0.2 µm) 10 times at 70°C. Then, a 100 mmol/L citrate buffer was added thereto to give a liposome suspension having a concentration of hydrogenated soybean phosphatidylcholine of 62.5 mg/mL. Separately, 10 mg of UCN-01 was taken and 8

mL of the liposome suspension prepared above was added thereto. The pH of the resultant mixture was adjusted to 8 by adding an appropriate amount of 1 mol/L aqueous sodium hydroxide. Then, distilled water was added thereto to give a total volume of 10 mL. The mixture was heated at 70°C for 5 minutes to thereby encapsulate UCN-01 in liposomes.

The average particle size of the liposomes measured by the DLS method was 130 nm.

#### Example 3

To 5 mL of the liposome suspension containing UCN-01 as prepared in Example 2 was added 0.05 mL of a 1.25 g/mL solution of PEG-DSPE {1,2-distearoyl-sn-glycero-3-phosphatidylethanolamine-*N*-(polyethylene glycol 2000); manufactured by Avanti} in ethanol. Then, the mixture was heated at 70°C for 2 minutes to thereby coat the surface of the liposomes with polyethylene glycol (PEG).

The average particle size of the liposomes measured by the DLS method was 136 nm.

#### Example 4

To 0.7 g of distearoyl phosphatidylcholine [DSPC, phase transition temperature: 58°C and 56°C (ed. by Shoshichi Nojima *et al.*, *Liposome*, p.77, 1988, Nankodo)] was added about 5 mL of a 100 mmol/L citrate buffer (pH 4.0), followed by shaking under stirring with a vortex

mixer. The suspension was passed through a polycarbonate membrane filter (0.4  $\mu\text{m}$ ) 10 times at 70°C, and further passed through a polycarbonate membrane filter (0.2  $\mu\text{m}$ ) 10 times at 70°C. Then, a 100 mmol/L citrate buffer was added thereto to give a liposome suspension having a DSPC concentration of 62.5 mg/mL. Separately, 5 mg of UCN-01 was taken and 4 mL of the liposome suspension prepared above was added thereto. The pH of the resultant mixture was adjusted to 8 by adding an appropriate amount of 1 mol/L aqueous sodium hydroxide. Then, distilled water was added thereto to give a total volume of 5 mL. The mixture was heated at 70°C for 5 minutes to thereby encapsulate UCN-01 in liposomes.

The average particle size of the liposomes measured by the DLS method was 180 nm.

#### Comparative Example 1

To 20 g of hydrogenated soybean phosphatidylcholine {phase transition temperature: 58°C (*FEBS Lett.*, 386, 247-251 (1996))} was added 70 mL of a 100 mmol/L citrate buffer (pH 4.0), followed by shaking under stirring with a vortex mixer. The suspension was passed through a polycarbonate membrane filter (0.4  $\mu\text{m}$ ) 4 times at 70°C, and further passed through a polycarbonate membrane filter (0.1  $\mu\text{m}$ ) 10 times at 70°C. Then, a 100 mmol/L citrate buffer was added thereto to give a liposome suspension having a

concentration of hydrogenated soybean phosphatidylcholine of 62.5 mg/mL. Separately, 20 mg of UCN-01 was taken and 16 mL of the liposome suspension prepared above was added thereto. The pH of the resultant mixture was adjusted to 8 by adding an appropriate amount of 1 mol/L aqueous sodium hydroxide. Then, distilled water was added thereto to give a total volume of 20 mL. The mixture was heated at 70°C for 5 minutes to thereby encapsulate UCN-01 in liposomes. After ice-cooling, 1.6 mL of the liposome suspension containing UCN-01 was taken and 6.4 mL of distilled water was added thereto. The resultant mixture was ultracentrifuged (25°C, 110,000 g × 1 hour), and 6.7 mL of the supernatant was removed. Then, distilled water was added to the precipitate, followed by re-suspending to give a UCN-01 concentration of 1 mg/mL.

The average particle size of the liposomes measured by the DLS method was 109 nm.

#### Comparative Example 2

To 15 g of yolk phosphatidylcholine [EggPC, phase transition temperature: -15 to -7°C (ed. by Shoshichi Nojima et al., *Liposome*, p.77, 1988, Nankodo)] was added 75 mL of a 100 mmol/L citrate buffer (pH 4.0), followed by shaking under stirring with a vortex mixer. The suspension was passed through a polycarbonate membrane filter (0.4 µm) 10 times at room temperature. Then, a 100 mmol/L citrate

buffer was added thereto to give a liposome suspension having an EggPC concentration of 62.5 mg/mL. Separately, 5 mg of UCN-01 was taken and 4 mL of the liposome suspension prepared above was added thereto. The pH of the resultant mixture was adjusted to 8 by adding an appropriate amount of 1 mol/L aqueous sodium hydroxide. Then, distilled water was added thereto to give a total volume of 5 mL. UCN-01 was encapsulated in liposomes at room temperature.

The average particle size of the liposomes measured by the DLS method was 274 nm.

#### Comparative Example 3

To 1.1 g of dipalmitoyl phosphatidylcholine [DPPC, phase transition temperature: 41°C and 35°C (ed. by Shoshichi Nojima et al., *Liposome*, p.77, 1988, Nankodo)] was added about 7 mL of a 100 mmol/L citrate buffer (pH 4.0), followed by shaking under stirring with a vortex mixer. The suspension was passed through a polycarbonate membrane filter (0.4  $\mu$ m) 15 times at 55°C, and further passed through a polycarbonate membrane filter (0.2  $\mu$ m) 10 times at 55°C. Then, a 100 mmol/L citrate buffer was added thereto to give a liposome suspension having a DPPC concentration of 62.5 mg/mL. Separately, 5 mg of UCN-01 was taken, and 4 mL of the liposome suspension prepared above was added thereto. The pH of the resultant mixture was adjusted to 8 by adding an appropriate amount of 1

mol/L aqueous sodium hydroxide. Then, distilled water was added thereto to give a total volume of 5 mL. UCN-01 was encapsulated in liposomes by heating the mixture at 55°C for 5 minutes.

The average particle size of the liposomes measured by the DLS method was 179 nm.

#### INDUSTRIAL APPLICABILITY

The present invention provides a method of inhibiting the leakage of a drug encapsulated in liposomes and a liposome preparation which is stable *in vivo*.

CLAIMS

1. A method of inhibiting the leakage of a drug encapsulated in liposomes in the presence of a biological component, which comprises using at least two lipid bilayers of the liposomes.

2. A method of inhibiting the leakage of a drug encapsulated in liposomes in the presence of a biological component, which comprises using lipid having a phase transition temperature higher than *in vivo* temperature as lipid constituting the liposomes.

3. A method of inhibiting the leakage of a drug encapsulated in liposomes in the presence of a biological component, which comprises satisfying at least two requirements selected from the group consisting of the following three requirements: using at least two lipid bilayers of the liposomes, controlling the average particle size of the liposomes to 120 nm or more, and using lipid having a phase transition temperature higher than *in vivo* temperature as lipid constituting the liposomes.

4. The method of inhibiting the leakage according to claim 2 or 3, wherein the lipid comprises at least one component selected from the group consisting of

hydrogenated soybean phosphatidylcholine, polyethylene glycol-modified phospholipid, and cholesterol.

5. The method of inhibiting the leakage according to claim 2 or 3, wherein the lipid comprises at least one component selected from the group consisting of distearoyl phosphatidylcholine, polyethylene glycol-modified phospholipid, and cholesterol.

6. A method of inhibiting the leakage of a drug encapsulated in liposomes in the presence of a biological component, which comprises using at least two lipid bilayers of the liposomes, and controlling the average particle size of the liposomes to 120 nm or more.

7. The method of inhibiting the leakage according to claim 3 or 6, wherein the liposomes have an average particle size of 120 to 500 nm.

8. The method of inhibiting the leakage according to any one of claims 1 to 7, wherein the biological component is a blood component.

9. The method of inhibiting the leakage according to any one of claims 1 to 8, wherein the drug encapsulated is an indolocarbazole derivative.

10. The method of inhibiting the leakage according to any one of claims 1 to 8, wherein the drug encapsulated is an antitumor agent.

11. The method of inhibiting the leakage according to any one of claims 1 to 8, wherein the drug encapsulated is an antibiotic.

12. The method of inhibiting the leakage according to any one of claims 1 to 8, wherein the drug encapsulated is a pharmaceutically active substance.

13. A liposome preparation in which the number of lipid bilayers of the liposomes is at least two, and the liposomes have an average particle size of 120 nm or more.

14. A liposome preparation in which the number of lipid bilayers of the liposomes is at least two, and lipid constituting the liposomes has a phase transition temperature higher than *in vivo* temperature.

15. A liposome preparation in which the liposomes have an average particle size of 120 nm or more, and lipid constituting the liposomes has a phase transition temperature higher than *in vivo* temperature.

16. A liposome preparation which satisfies at least two requirements selected from the group consisting of the following three requirements: the number of lipid bilayers of the liposomes is at least two, the liposomes have an average particle size of 120 nm or more, and lipid constituting the liposomes has a phase transition temperature higher than *in vivo* temperature.

17. The liposome preparation according to any one of claims 13 to 16, which inhibits the leakage of a drug encapsulated in the liposomes in the presence of a biological component.

18. The liposome preparation according to claim 17, wherein the biological component is a blood component.

19. The liposome preparation according to any one of claims 14 to 18, wherein the lipid comprises at least one component selected from the group consisting of hydrogenated soybean phosphatidylcholine, polyethylene glycol-modified phospholipid, and cholesterol.

20. The liposome preparation according to any one of claims 14 to 18, wherein the lipid comprises at least one component selected from the group consisting of

distearoyl phosphatidylcholine, polyethylene glycol-modified phospholipid, and cholesterol.

21. The liposome preparation according to any one of claims 13 and 15 to 18, wherein the liposomes have an average particle size of 120 to 500 nm.

22. The liposome preparation according to any one of claims 13 to 21, wherein the drug encapsulated is an indolocarbazole derivative.

23. The liposome preparation according to any one of claims 13 to 21, wherein the drug encapsulated is an antitumor agent.

24. The liposome preparation according to any one of claims 13 to 21, wherein the drug encapsulated is an antibiotic.

25. The liposome preparation according to any one of claims 13 to 21, wherein the drug encapsulated is a pharmaceutically active substance.

**COMBINED DECLARATION AND POWER OF ATTORNEY  
FOR PATENT COOPERATION TREATY APPLICATION**

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled METHOD OF INHIBITING LEAKAGE OF DRUG ENCAPSULATED IN LIPOSOMES, the specification of which was filed as PCT International Application No. PCT/JP00/04140 on 23.06.00 and was amended under PCT Article 19 on \_\_\_\_\_ (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, § 119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) on which priority is claimed:

Country	Application No.	Filed (Day/Mo./Yr.)	Priority Claimed (Yes/No)
Japan	178142/99	24 June 1999	Yes

I hereby appoint the practitioners associated with the firm and Customer Number provided below to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith, and direct that all correspondence be addressed to the address associated with that Customer Number:

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COMBINED DECLARATION AND POWER OF ATTORNEY  
FOR PATENT COOPERATION TREATY APPLICATION  
(Page 2)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

1. Full Name of Sole or First Inventor Yasuki Kato

Inventor's Signature Yasuki Kato

Date November 22, 2001 Citizen/Subject of JAPAN

Residence Susono-shi, Japan JPY

Post Office Address c/o Pharmaceutical Research Institute, KYOWA HAKKO KOGYO CO., LTD., 1188, Shimotogari, Nagaizumi-cho, Sunto-gun, Shizuoka 411-8731 Japan

2. Full Name of Second Joint Inventor, if any Masahiro Yamauchi

Inventor's Signature Masahiro Yamauchi

Date November 22, 2001 Citizen/Subject of JAPAN

Residence Sunto-gun, Japan JPY

Post Office Address c/o Pharmaceutical Research Institute, KYOWA HAKKO KOGYO CO., LTD., 1188, Shimotogari, Nagaizumi-cho, Sunto-gun, Shizuoka 411-8731 Japan

3. Full Name of Third Joint Inventor, if any Hiroko Kusano

Inventor's Signature Hiroko Kusano

Date November 22, 2001 Citizen/Subject of JAPAN

Residence Sunto-gun, Japan JPY

Post Office Address c/o Pharmaceutical Research Institute, KYOWA HAKKO KOGYO CO., LTD., 1188, Shimotogari, Nagaizumi-cho, Sunto-gun, Shizuoka 411-8731 Japan

4. Full Name of Fourth Joint Inventor, if any Atsushi Ishihara

Inventor's Signature Atsushi Ishihara

Date November 22, 2001 Citizen/Subject of JAPAN

Residence Sunto-gun, Japan JPY

Post Office Address c/o Pharmaceutical Research Institute, KYOWA HAKKO KOGYO CO., LTD., 1188, Shimotogari, Nagaizumi-cho, Sunto-gun, Shizuoka 411-8731 Japan